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Small Molecule Directed Differentiation of Pluripotent Stem Cells and the Outcome on Cardiogenesis.

A Thesis

Submitted to the Faculty

Of

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By

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of

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ABSTRACT

Small Molecule Directed Differentiation of Pluripotent
Stem Cells and the Outcome on Cardiogenesis.
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Though the heart is one of the first organs to develop during embryogenesis and the physical aspects of development are well documented, little is known of the molecular mechanisms that control heart development. BMP signaling has been implicated in cardiac development both *in vivo* and *in vitro*, the initial research focused on altering this pathway. BMP signaling belongs to the signaling superfamily of transforming growth factor-b (Tgf-β). Further evidence from mouse knockout studies, reveals a critical role of signaling through the Tgf-β receptors in which Tgf-β 3−/− mice demonstrate congenital heart defects. Tgf-β signaling is typically relayed through a tetramer complex composed of two Tgf-β type II and two type I (ALK5) receptors. The signaling of this tetramer has recently been identified in the differentiation of epicardial and endocardial to mesenchyme. Proceeding experiments have demonstrated that knocking ALK5 out selectively in endocardium, myocardium, or epicardium does not interfere with normal cardiac muscle development *in vivo*. Sridurongrit suggest that ALK5 signaling is required for smooth muscle development and vascularization of the myocardium but not cardiomyocoyte development. Therefore the role of ALK5 signaling during cardiac development is studied int two pluripotent models, mouse embryonic stem cells and human induced pluripotent stem cells (hiPS) in this research to understand the role of this pathway in cardiogenesis. Further the ultimate goals of this research is to screen small molecules and develop protocols that direct diffentiation of pluripotent stem cells to mesoderm and ultimately a cardiomyocyte fate.

There are two major differentiation events that occur as a pluripotent stem cell differentiates to a terminal state. The cell begins as a pluripotent cell that can give rise to all

somatic cell types as this cell differentiates it enters multipotent stage. Multipotent cells become partially programmed and can give rise to only certain somatic fates. These multipotent progenitors will ultimately give rise to structured tissue composed of specific somatic cell types. However, the molecular pathways that control differentiation to specific somatic fates remain poorly understood. The focus of this research is to explore these pathways using small molecule inhibitors to better understand the internal cell signaling that controls cardiogenesis. The research presented in this paper occurs in two major stages. First the experiments focus on developing protocols that can induce pluripotent stem cells to give rise to mesoderm, the germ layer from which cardiomyocytes are derived. Secondly, small molecules are screened to understand their ability to drive this mesoderm to a cardiomyocyte fate.

Exploring these pathways, that control cardiogenesis, is essential if stem cells are to provide a supply of primary cardiomycotes to better understand human cardiac physiology and the affect potential drugs will have on their function. Heart disease remains the number one cause of death in the developed world. Therefore there is not only a need to develop novel molecules that can assuage cardiac disease but there is also a need to understand how these diseases develop. hiPS have the potential to fulfill both these needs. These cells can be derived directly from patients with specific cardiac afflictions. By controlling the differentiation of these disease derived pluripotent cells, researchers will be able to track physical and chemical changes in cardiomyocyte development that ultimately lead to a diseased phenotype. This creates a powerful tool to study new molecules and cardiac disease. Screening of small molecules that alter the diseased phenotype of these patients will further understanding of chemical modulation of cardiomyocytes and the ability of potential drugs to mitigate disease. This research has the potential to ultimately lead to patient specific therapeutics in the treatment of heart disease.

CHAPTER 1: BACKGROUND

Current functional assays in the pharmaceutical industry heavily rely on the use of immortalized cells lines artificially expressing a target of interest. Immortal cell lines are not representative of normal signaling pathways that are found endogenously. These cells often contain abnormal karyotypes because they are derived from tumors. Many are also not of human origin. This creates an assay system that is misleading and consumes valuable time and resource to establish which drugs are truly interacting with the target and which are merely noise from assay interference created by the cell line (Puton *et al*, 2007). Consequently primary cell cultures that provide genetically normal cells would be preferred; however these cells do not maintain in culture for extended periods of time and lose their functional systems as they dedifferentiate. Since primary cells are from mature tissue, it is not possible to obtain human heart cells in quantity (Puton *et al*, 2005). Currently large Pharmaceutical and Biotech companies screen millions of compounds per drug target making primary cell culture not feasible. Breakthrough work with stem cells has provided an alternative path; these cells remain in culture indefinitely and can differentiate into all somatic cell types. However, the pathways that control differentiation to specific somatic fates are not well understood. Therefore there is a need within the scientific community to develop robust differentiation protocols to produce mature cell types for understanding disease and drug discovery.

Considerable progress has been made in the scientific field that allows for the characterization of human embryonic stem cells (hES). These cells have the ability to not only self-regenerate but also differentiate into all somatic cell types from neurons to endothelial cells to cardiomyocytes (Koestenbauer *et al*, 2006). However, the source of hES, the blastocyst, is a topic for ethical debate. A recent milestone discovery, human induced pluripotent stem cells (hiPS), has provided a way to avoid these ethical considerations. hiPS are adult somatic cells that have been returned from a state of terminal differentiation to a state of pluripotency through the

ectopic expression of transcription factors, *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*. hiPS creation requires only basic knowledge of molecular biology and does not require specialized equipment, therefore their production is efficient and not costly(Ohnuki *et al*, 2009). More importantly hiPS have not only shown to be pluripotent but to possess highly similar epigenetic characteristics of the hES genome. Endogenous genes related to pluripotency, such as *Oct4* and *Nanog*, become hypomethylated in hiPS and histone modifications of developmental regulators were returned to a bivalent state as they are observed in hES (Werning *et al*, 2007). Thus, hiPS have the potential to provide a tool for assay technology and disease research with all the benefits of hES models.

One of the most costly stalling points in the current drug discovery process is the induction of adverse cardiac effects by small molecule therapeutics (Kettenhofen *et al*, 2008). This provides a palpable goal for understanding how to generate mature cardiomyocytes from hiPS. There are two main areas that the generation of cardiomyocytes from hiPS could be applied to in the drug discovery continuum, safety assessment and cardiac therapeutics.

The first area would be to screen for the safety assessment of novel small molecules in mature cardiomyocytes. Given that pharmaceutical companies are required by regulatory agencies to explore metabolites generated by cytochrome p450 enzymes, one could potentially screen the novel drug and all its metabolites against cardiomyocytes to observe disruptions in normal electrophysiological function. Understanding a drug's potential for delaying the repolarization of the cardiac ventricular action potential has become a major focus of pharmaceutical companies. The driving force of this lies in the tendency of new compounds to possess an effect on prolongation of the QT interval which leads to cardiac arrhythmia (Shah, 2002). QT prolongation in the last decade has become a major cause of drug withdrawal (Sartipy *et al*, 2007). If companies had a reliable source of ventricular cardiomyocytes, it would be simple to screen for QT prolongation. Standard path clamp techniques can be employed to measure the effects a compound has on the electrophysiological properties of ventricular cardiomyocytes (Mummery *et*

al, 2003). Large colonies of cardiomyocytes can also be used to understand how a compound affects the rhythm, origin of excitation, conduction, and repolarization of cardiomyocytes by placing these colonies onto Micro Electrode Arrays. Reppel *et al*. have already demonstrated that the delay in repolarization caused by s-sotalol can be observed using this method (Reppel *et al*, 2005). Therefore, hiPS differentiated into cardiomyocytes reliably and in quantity could alter the future of compound cardiac safety assessment.

The second area to apply cardiomyocyte screening would be the area of understanding pharmacological modulation of cardiomyocytes' function in diseases where the heart is the diseased organ. Readily available access to human cells will serve as models for target identification and validation. This could greatly increase the chances of discovering novel therapeutics because animal models or transfected cells lines simply cannot provide the human physiologic environment of primary cell culture. Moreover, pluripotent cells could be altered genetically before differentiation to represent genetic disorders, such as congenital long QT syndrome, providing a model for studying not only disease development throughout differentiation but also targets for pharmacological intervention. Cardiomyocytes derived from hiPS will therefore provide an *in vitro* model for cellular responses in disease such as but not restricted to cardiac arrhythmia, contractile function, response to oxidative stress, and protection from ischemia (Sartipy *et al*, 2007).

Several criteria must first be met before developing a truly predictive cardiac model. Firstly, developing a protocol that would give rise to a homogeneous population of cardiomyocytes from pluripotent cells would be preferred. It would also be required that these cells possess endogenous signaling and electrophysiological properties found in endogenous human cardiomyocytes. Providing markers, such as fluorescent reporters for cardiac genes, would also provide a potent tool for following molecular events during differentiation. Lastly, a high throughput model for culture must be developed that allows for large scale creation of

cardiomyocytes from pluripotent cells for screening small molecules that can assuage cardiac disease (Beqqali, 2009). The goal of this current research will be to focus on this last criterion, developing methods that can contribute to a high yielding differentiation protocol of cardiomyocytes.

Understanding the molecular pathways that direct differentiation in pluripotent cells to cardiomyocytes will provide an alternative *in vitro* model that is more human relevant for elucidating the development of cardiac cells and how diseases may arise that affect them (Puton *et al*, 2005). However, using pluripotent cells to develop mature cardiomyocytes has proven difficult due to lack of knowledge of the mechanisms that control differentiation paths. Recent work has indicated that cardiogenesis happens through a series of steps of lineage commitments, that occur through such genes as *Nkx2.5* (Kouskoff *et al*, 2005). Other work has also shown simple "cardiac like" culture conditions are not appropriate to induce successful cardiogenesis in high yields (Wu *et al*, 2004). Therefore temporal modulation of signaling pathways through chemical intervention could prove to be the most efficient way to increase yields of cardiomyocytes from pools of hiPS.

CHAPTER 2: INTRODUCTION

The use of small molecules to direct cardiogenesis is novel from traditional transcription factor treatments. There are several positive outcomes that could be identified from indiscriminately screening small molecule agonists and antagonists in pluripotent cells for their ability to increase cardiogenesis. Foremost this method could identify pathways involved in signaling that control cardiogenesis that have not been implicated or understood in previous works. Further small molecules can be used to temporally alter signaling, are reversible, and can be broad based acting which has advantages over more specific inhibition methods such as siRNA (Willems *et al*, 2009). Therefore a major component of this research was to develop assays that allow for the screening of small molecules that could direct the differentiation of both mES and hiPS to a cardiomyocyte fate.

There are two major differentiation events that occur as a pluripotent stem cell differentiates to a terminal state. First the cell begins as a pluripotent cell that can give rise to all somatic cell types, around days four to six in the manual differentiation process the cell takes on a multipotent fate. These cells become partially programmed and can give rise to only certain somatic fates. These multipotent progenitors become terminally differentiated to a specific somatic fate by approximately day ten (Hochedlinger *et al*, 2009). Given that there are two main stages of differentiation, this presents us with an opportunity to optimize two distinct protocol at different time points to increase cardiomyocyte production from hiPS.

From days one to five, one can screen for molecules and test culture methods that lead to cardiac mesodermal precursors. By increasing the amount of precursor cells, the probability of receiving higher yields of cardiomyocytes also increases. However, this research is not without challenge. Though the heart is one of the first organs to develop during embryogenesis and the physical aspects of development are well documented, little is known of the molecular mechanisms that control heart development (Sartipy *et al*, 2007).

Recent studies have begun to reveal several major genes identified in cardiac development from model organism studies, such as *Drosophila melanogaster, MEF2, Isl-1* and *Nkx2.5* (Moretti *et al*, 2006 & Olson 2004). Time course studies *in vitro* in pluripotent models reveal *Isl-1* mRNA or its functional protein is not expressed in early embryoid bodies. After four to six days of differentiation *Isl-1* expression can be detected in a subset of these cells (Moretti *et al*, 2006). Sun et al. have demonstrated that most *Isl-1* progenitor cells have migrated to the region of heart development during mouse embryonic development. This region gives rise to multiple cardiovascular cells including cardiomyocyte, endothelial, and smooth muscle therefore suggesting *Isl-1* positive cells are the cardiac progenitor cells (Sun *et al*, 2007). A model proposed by Chien *et al*. suggests *Isl-1* positive cells eventually express two other genes, *Nkx2.5* and *flk1*, which serve as the primordial cardiac progenitors that give rise to cardiac muscle, smooth muscle, and endothelial cells. However, *Isl1* and *flk1* positive cells gave rise to endothelial cells but *Isl-1* and *Nkx2.5* positive cells ultimately generate either cardiac or smooth muscle lineages (Moretti *et al*, 2006). During this cascade of transcription factors, other research has demonstrated that the MADS-box myocyte enhancement factor-2 (*MEF2*) plays a crucial role in coordinating these transcription factors to activate genes associated with early cardiac mesoderm and production of proteins that compose the myofibrils of cardiac muscle (McKinsey *et al*, 2002). Therefore, the first experimental model used in this research, mES, has been constructed with a reporter vector for *MEF2C* driving dsRed. If early tested culture conditions are truly forming cardiac mesoderm, this reporter should be detectable during differentiation experiments around day five.

Further exploration of signaling during early differentiation of hiPS is also explored in this research. The constituents of the bone morphogenetic protein (BMP) family have been identified to contribute to cardiac mesoderm development (Schultheiss *et al*, 1997). BMP signaling studies have revealed their involvement in up regulating several of the previously

mentioned transcription factors, most notably *Nkx2.5* (Brand, 2007). Knockout studies performed in mice, have also displayed clear developmental heart abnormalities in BMP2 -/- mice (Zhang *et al*, 1996). Canonical Wnt signaling, such as Wnt3A, and fibroblast growth factors have also been linked to early cardiogenesis through inhibition studies. Disrupting the ligands for these three signaling families have all shown to have reproducible disturbances in cardiac development, which has been conserved across various species (Olsen *et al*, 2003). The role of BMP4 and Activin A signaling are also directly associated with early mammalian mesoderm development which is essential to cardiac development (Johansson *et al*, 1995). Therefore, combinations of Wnt3, Activin-A, BMP2, and BMP4 were all explored in this study during early differentiation of hiPS to determine their ability to increase cardiomyocyte production. Ultimately to contribute to the development of an optimal 2-d culture system for differentiation of cardiac tissue from hiPS.

Secondly, one can screen from days 6 to 10, to identify compounds that drive multipotent precursor to mature cardiomyocytes. Around day ten, there are two genes that have been demonstrated to be canonical cardiac genes, cardiac Troponin T and α-Myosin Heavy chain (Martin-Puig, 2008). If compounds are truly inducing cardiogenesis, the expression of both these genes should be up-regulated and tractable.

The research presented in this paper explores this second half of mES and hiPS differentiation as well, by testing several small molecule inhibitors of signaling pathways that have been identified in differentiation. Given that BMP signaling has been implicated in cardiac development both *in vivo* and *in vitro*, the initial research focused on altering this pathway. BMP signaling belongs to the signaling superfamily of transforming growth factor-b (Tgf-β) (Wagner *et* al, 2007). Further evidence from mouse knockout studies, reveals a critical role of signaling through the Tgf-β receptors in which Tgf-β 3−/− mice demonstrate congenital heart defects (Kaartinen *et* al, 1995). Tgf-β signaling is typically relayed through a tetramer complex composed of two Tgf-β type II and two type I (ALK5) receptors. The signaling of this tetramer has recently

been identified in the differentiation of epicardial and endocardial to mesenchyme. Proceeding experiments have demonstrated that knocking ALK5 out selectively in endocardium, myocardium, or epicardium does not interfere with normal cardiac muscle development *in vivo*. Sridurongrit suggest that ALK5 signaling is required for smooth muscle development and vascularization of the myocardium but not cardiomyocoyte development. The work suggests down regulating ALK5 leads to the activation of an alternate pathway of Tgf-β signaling that could increase myocardium development (Sridurongrit *et al*, 2008). It was therefore a hypothesis of this research that inhibiting ALK5 signaling after mesoderm induction could increase the percentage of cardiomyocytes formed from pluripotent cells. After validating this theory in a mES cell model, further experiments were developed to test this hypothesis in hiPS and to test alternate pathways.

CHAPTER 3: PROOF OF CONCEPT

Studies have demonstrated that screening for small molecules that stimulate or inhibit differentiation paths may possess promise for greater success of differentiation control. Hao *et al.* demonstrated that application of Dorsomorphin, a bone morphogenetic protein (BMP) inhibitor, during the initial stages of stem cell differentiation significantly promoted differentiation toward cardiomyocytes at the expense of other mesodermal lineages (Hao *et al*, 2008). Small scale screens of kinase inhibitors have also yielded success in increasing cardiomyogenesis from stem cells. Graichen *et al*. identified a p38 MAP kinase specific inhibitor that induced more than 20% of differentiated cells to cardiomyocytes and also increased the yield of these cells by 2.5 over the control population (Graichen *et al*, 2008).

More recently, Berkessel *et al.* have related similar characteristics of stem cells and cancer cells with regards to their ability to rapidly regenerate. To explore their theory they hypothesized that drugs that induce differentiation will also slow their regeneration rate. Thus, anticancer therapeutics that reduce cell division were explored to induce cardiogenesis. The screen revealed four compound derivatives of the prominent anti cancer drug, Nexavar, increased their green fluorescent protein reporter system 50 to 80% higher than their control populations. More importantly, the study revealed time dependent studies do have an effect on the positive outcome of cardiogenesis (Berkessel *et al*, 2010). Thus, temporal expression of genes will have to be explored when screening for small molecules that induce cardiogenesis.

Pertinent to developing a new model for safety assessment and drug discovery, studies have also shown that cardiomyocytes developed from human pluripotent cells through differentiation protocols exhibit many of the electrical and physiological properties of mature cardiac cells (He *et al*, 2003). Therefore, a protocol providing reliable cardiac differentiation would allow for clinically relevant assays of novel compounds to determine disruption of cardiac

electrophysiological properties before animal model testing (Beqqali *et al*, 2009). As a consequence, these primary cell assays will not only save time and money in the drug discovery process but theoretically possess the possibility of increasing the ethics of drug discovery by decreasing the number of model organisms needed to validate a drug's safety.

CHAPTER 4: MATERIAL AND METHODS

4.1 Cell Medias

MEF Cell Culture Media:

Minimum Essential Medium Earl's w/ High Glucose & No Phenol Red - Gibco

- 10% FBS - Gibco
- 1x Glutamax - Gibco
- 1x Pen/Strep - Invitrogen
- 1x Non essential Amino Acids – Gibco

mES Cell Culture Media:

DMEM w/ High Glucose w/ sodium bicarbonate

- 10% FBS - Gibco
- 1x Glutamax - Gibco
- 1x Pen/Strep - Invitrogen
- 1x Non essential Amino Acids - Gibco
- 1x Sodium Pyruvate 100mM - Gibco
- 5uL of 2-Mercapto-Ethanol(BME) for 500mL of media – Sigma Aldrich

*Cell media was supplemented with 4ng/mL of LIF Recombinant Human (Invitrogen), reconstituted in PBS and 10% BSA (Sigma-Aldrich), to maintain pluripotency and prevent differentiation.

hIPS maintenance media

Gibco Knockout Media DMEM F12 – Lot 12660-012

- 20% Gibco Knockout SR – 10828-028

- 20ng/mL BFGF - Invitrogen
- 1x Pen/Strep - Invitrogen
- 1x Glutamax - Gibco
- 4uL/500mL 2-Mercaptoethanol(BME) - Sigma-Aldrigh

hiPS Matrigel Media – mTESR

mTESR-1 Basal Medium - Wicell Cat #05850

- mTESR 5X Supplement - Wicell
- 1x Pen/Strep - Invitrogen
- 1x Glutamax - Gibco

hIPS Differentiation Media – StemPro®-34 Serum-free Medium

StemPro34 – Invitrogen Cat #10639-011

- 1x Pen/Strep - Invitrogen
- 1x Glutamax - Gibco

4.2 Antibodies

Mouse Cardiac Troponin T – clone 1C11 – Abcam – ab8295, primary

Rabbit Cardiac Troponin T – Invitrogen – A21202, primary

Goat Anti-Rabbit - Invitrogen – A21245, secondary

Donkey Anti-Mouse – Invitrogen – A21202, secondary

4.3 Pluripotent Cell Lines

Mouse Embryonic Stem Cells – mES4 line with MEF2C reporter – Harvard Stem Cell Institute

Human Induced Stem Cells – hiPS line derived from human fibroblasts – Harvard Stem Cell Institute

4.4 Culture of Mouse Embryonic Stem Cells(Prior to differentiation)

On day one of the mES experiments, petri dishes that have been culture treated were coated with gelatin(Millipore Ultrapure 0.1% Gelatin). In order to gelatinize the plates, 5mL of the gelatin solution was dispensed onto a 10mL culture dish (Corning). This was allowed to incubate for 1 hour at 37°C. After the incubation step, the plate was removed from the incubator and gelatin solution was removed and replaced with fresh MEF media (Medias outlined above).

24 hours prior to thawing mES, mitomycin C inactivated MEFs (Millipore) were plated onto a gelatinized 10mL cell culture plate. MEFs were thawed in warm water. A 1mL vial of MEFs was brought up in 10mL MEF media and spun down for 4 minutes at 1000 rpms to remove freezing media DMSO. MEFs were resuspended at 1 million cells per 1 mL. 1 mL was then added to the gelatinized petri dish with 11mL MEF media (1 million MEFs/plate). Cells were incubated 24 hours at 37C and 5%CO2.

The following day, a 1 mL vial of mES cells (Havard Stem Cell Institute) was thawed in warm water. Cells were brought up in 10 mL mES media and spun down for 4 minutes @1000 rpm. Freezing media was removed and cells were resuspended in mES media supplemented with 4ng/mL LIF (Invitrogen). The LIF supplementation prevents random differentiation. MEF media was aspirated from MEF plated cell culture dish and mES cells constituted in mES media/LIF were plated onto MEFs. Cells were fed fresh media every 24 hours until they reached confluency

or began to differentiate. mES were re-plated onto a second passage of fresh MEF culture. Following a second passage of confluency, the cells were passaged onto 10mL cell culture dishes that were gelatinized prior to passage. Passage onto gelatinized plates was repeated before differentiation experiments were performed. Passages onto gelatinized plates without MEFs were repeated for two to three passages to ensure MEFs are not present when differentiation experiments begin.

4.5 Differentiation of mES by Embryoid Body(EB)

mES cells that were grown on gelatin were washed once with cold 0.05% Trypsin solution (Gibco). Cells were then trypsinized with cold 0.05% Trypsin for 3 minutes at room temperature. Cells were triturated in order to create single cell suspension. Cells were collected in mES media without LIF. Cells were counted and diluted to 75,000 cells/mL or 100,000 cells/mL. Hanging suspensions were created by plating 20uL drops on the bottom of a cell culture dish then inverting the plates. This created ~1500 cell EBs or ~2000 cell EBs, respectively. Cells were incubated for 24 hours in inverted suspension. On day 2 of the experiment, after visual confirmation of EB formation, plates were flipped and cells were flooded with fresh mES media. Cells were fed daily with fresh media throughout the EB stage.

4.6 Plating of Dissociated EBs and Whole EBs

On day 6, EBs were collected from the cell culture dish and pooled together. EBs were spun down for 4 minutes @ 500 rpm. Supernatant is aspirated and cells were washed once with PBS. Cells were re-spun for 4 minutes at 350 rpm. 4/5 of the cells were collected to be dissociated, the other 1/5 was plated with ~1 to 2 EBs per well of a tissue culture treated 96 well plates with flat bottom (Greiner). The EBs, to be dissociated, were incubated in 0.05% Trypsin for 5 minutes at 37C. After incubation cells were triturated to break up any remaining clumps. Cells were then plated at 3K, 10K, and 20K cells/well of a 96 well plate. Cells were plated in mES media at 100uL/well. Cells were fed each day, with old media aspirated and 100uL of fresh

mES media added. Cells were cultured in 96 well plates until desired time point was reached for quantification of differentiation markers.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100nM	100nM	300nM	300nM	1.0 uM	1.0 uM	3uM	3uM	Vehicle	Vehicle	Vehicle	Vehicle
B	100nM	100nM	300nM	300nM	1.0 uM	1.0 uM	3uM	3uM	Vehicle	Vehicle	Vehicle	Vehicle
C	100nM	100nM	300nM	300nM	1.0 uM	1.0 uM	3uM	3uM	Vehicle	Vehicle	300nM	300nM
D	100nM	100nM	300nM	300nM	1.0 uM	1.0 uM	3uM	3uM	Vehicle	Vehicle	300nM	300nM
E	100nM	100nM	300nM	300nM	1.0 uM	1.0 uM	3uM	3uM	Vehicle	Vehicle	1.0 uM	1.0 uM
F	100nM	100nM	300nM	300nM	1.0 uM	1.0 uM	3uM	3uM	Vehicle	Vehicle	1.0 uM	1.0 uM
G	100nM	100nM	300nM	300nM	1.0 uM	1.0 uM	3uM	3uM	Vehicle	Vehicle	3uM	3uM
H	100nM	100nM	300nM	300nM	1.0 uM	1.0 uM	3uM	3uM	Vehicle	Vehicle	3uM	3uM

3K cells/well
10K cells/well
20K cells/well
Embroid Body

Figure i. Design of mES ALK5 inhibitor experiment design.

4.7 Drug Treatment of mES

mES that were re-plated into 96 well plates were treated with compounds on day 7. (Observe design for ALK5 inhibitor dosing in Figure i). Compounds were diluted to desired concentration in mES cell culture media without LIF to a total DMSO (Sigma-Aldrich) concentration of 1%. Vehicle controls were composed of 1% DMSO diluted in mES cell culture media without LIF. 1mL of compound solution media or 1 mL of vehicle control was added to each test well. Compounds were allowed to incubate for 3 days and removed on day 9. Drug treated mES and controls were washed twice with mES cell culture media without LIF and each well received 1 mL of fresh media. mES were fed daily throughout the entire differentiation experiment with mES cell culture media without LIF.

4.8 Culture of Human Induced Pluripotent Stem Cells(Prior to Differentiation)

On day one of the hiPS experiments, cell culture dishes (Corning) that have been culture treated are coated with gelatin(Millipore Ultrapure 0.1% Gelatin). In order to gelatinize the plates, 5mL of the gelatin solution was dispensed onto a 10mL culture dish. This was allowed to incubate for 1 hour at 37°C. After the incubation step, the plate was removed from the incubator and gelatin solution was removed and replaced with fresh MEF media.

24 hours prior to thawing hiPS, mitomycin C inactivated MEFs were plated onto a gelatinized 10mL cell culture plate. MEFs were thawed in warm water. A 1mL vial of MEFs was brought up in 10mL MEF media and spun down for 4 minutes at 1000rpms to remove freezing media DMSO. MEFs were resuspened at 1 million cells per 1 mL. 1 mL was then added to the gelatinized cell culture dish with 11mL MEF media (1 million MEFs/plate). Cells were incubated 24 hours at 37C and 5%CO2.

The following day a 1mL vial was thawed of hiPS derived from human fibroblasts (Harvard Stem Cell Institute). The hiPS were diluted in hiPS maintenance media at room temperature in a 50mL conical (BD Sciences). The hiPS were spun to pellet at 500 RPM for 3 minutes. While the hiPS were being spun down, MEFs were washed two times with hiPS maintenance media. hiPS were then re-suspended in maintenance media. hiPS were counted and diluted to 250K cells/mL. 1 mL was added to a plate of MEFs and brought up to a total volume of 12 mL of maintenance media. Throughout the culture process hiPS maintenance media was removed daily and replaced with a fresh 12 mL of maintenance media.

4.9 Passaging of hiPS

MEFs were plated 24 hours prior to passaging hiPS. On the day of passaging, MEFs were washed 2 times with hiPS maintenance media. hiPS were washed with cold 0.05% Trypsin (Gibco), one time. 4mL of cold 0.05% trypsin was added to each 10mL culture dish of confluent hiPS and incubated for 3 minutes at room temperature. Trypsin was aspirated after 3 minute incubation. hiPS were dissociated from culture plate using a stream of hiPS maintenance media. Cells were not broken up into single suspensions, colonies were left intact. hiPS were split into desired ratio and plated onto the 24 hour incubated MEFs in 12mL of maintenance media.

4.10 Plating hiPS onto Matrigel

BD Matrigel matrix (BD Biosciences) was partially thawed in hand before placing in 4°C to completely thaw. Matrigel was reconstituted at the recommended manufacturers concentration.

The thawed matrigel was diluted right away in mTESR media. 8mL of the Matrigel solution was added to 10cm culture plates (Corning). The plates were then allowed to incubate for a ½ hour at 37°C. hiPS were washed once with cold 0.05% Trypsin. 4mL of cold 0.05% trypsin was added to each 10mL culture dish of confluent hiPS and incubated for 3 minutes at room temperature. Trypsin was aspirated after 3 minute incubation. Colonies of hiPS were dissociated with a stream of mTESR. Cells were plated onto the fresh Matrigel in 12mL of mTESR. mTESR media was replaced daily with a fresh 12 mL/ 10cm culture plate.

4.11 Induction of hiPS for Mesoderm (Days 0 to 5 of Differentiation)

On day 0 of the differentiation experiment hiPS on Matrigel were washed with StemPro34, two times. A solution of Activin A (AA, Recombinant Human from Invitrogen) & BMP-2 (BMP2, Recombinant Human from Invitrogen) was made up at 10ng/mL in StemPro34. This solution was added to confluent colonies of hiPS on matrigel. Days 1 to 4 of the experiment, repeated the addition of AA/ BMP2 solution at a concentration of 40ng/mL after aspirating out previous day's solution. On day 5 of mesoderm induction, the previous day's solution was not aspirated and 3mL of fresh StemPro34 media is added. On day 6 cells are incubated with fresh media or were selected for lenti viral reporter transduction, cTnT reporter driving luciferase. On day 6 of the differentiation experiments, hiPS were re-plated onto 96 well plates (Greiner).

4.12 Plating hiPS 96 Well Plate

hiPS, that were induced for mesoderm on Matrigel, were washed with cold 0.05% Trypsin (Gibco). hiPS were then incubated with cold 0.05% Trypsin for 3 minutes. The trypsin was aspirated prior to dissociating the cells with StemPro34. The cells were gently pipetted up and down to break up colonies but single cell suspension was not formed or desired. Cell suspension was counted and re-suspended in StemPro34 at 5K cells/mL. 1mL of cell suspension was plated into 96 well culture plate (Greiner) that had been gelatinized (Millipore Ultra Pure Gelatin) 12 hours prior. Cells were fed daily with fresh StemPro34 media.

4.13 Drug Treatment of hiPS

hiPS that were re-plated into 96 well plates were treated with compound on day 7. Compounds were diluted to desired concentration in StemPro34 media with a total DMSO (Sigma-Aldrich) concentration of 1%. Vehicle controls were 1% DMSO diluted in StemPro34. 1mL of compound solution or 1 mL of vehicle control was added to each test well. Compounds were allowed to incubate for 3 days and removed on day 9. Drug treated hiPS were washed twice with StremPro34 and each well received 1 mL of fresh StemPro34. hiPS were fed daily throughout the entire differentiation experiment with StemPro34.

4.14 Fixing and Antibody Staining

Cells were fixed at various time points of the differentiation experiments (in days). Briefly, media was aspirated from the wells and each well was washed with PBS. 3.7% Paraformaldehyde (PFA from Sigma-Aldrich) solution in PBS was then added, 100uL/well. Cells were incubated at room temperature for 20 minutes. PFA solution was removed and cells were washed with PBS. Fixed cells were stored in PBS at 4C until permeabilization and staining. Cells were permeabilized for one hour with a Permeabilization/Blocking buffer: 70% Superblock in TBS (Thermo Scientific), 30% PBS, 0.1% TritonX (Promega), and 0.05% Tween20 (Promega). Cells were then incubated with primary antibody in 70% Superblock, 30% PBS, and 0.05% Tween20, overnight at 4C. Cells were washed with a solution of 70% PBS and 30% Superblock before secondary antibody addition. Secondary antibody in 70% Superblock, 30% PBS, and 0.05% Tween20, was added to each plate and incubated for 1 hour at room temperature. Cells were washed with a solution of PBS with 0.02% Hoechst stain (Thermo Scientific) after secondary antibody addition. 100uL of PBS with 0.02% Hoechst are then added before high content imaging with an Operetta (Perkin Elmer).

4.15 Lentivirus Production

A lentivirus reporter system was created to detect Cardiac Tropnonin T (cTnT) by luciferase detection. A vector was constructed from an in house vector that contained cTnT driving a GFP reporter. The GFP cassette was removed and replaced with a luciferase cassette by employing standard cloning techniques. The newly constructed vector was transferred into the HEK293T™ cell line from Open Biosystems. The packing mix was added and the lentiviral supernatant production was followed according to the manufacturer (Open Biosystems). The supernatant was collected, but lentivirus was not concentrated. The supernatant was used to transduce iCell cardiomyocytes(CDI) to determine lentiviral efficiency. Transduction percentages are calculated volume to volume, lentivirus to cell media.

4.16 Real Time Quantitative Reverse Transcription PCR (qRT-PCR)

Cells were lysed with 100uL Buffer RLT (Qiagen). RNA was isolated following the protocol of RNeasy kit (Qiagen). Samples were reverse transcribed using the High Capacity cDNA Kit (Applied Biosystems). Reverse transcription reactions were carried out for 2 hours at 37°C. Primers for genes were obtained externally (Applied Biosystems). PCR was performed on MasterCycler EP RealPlex (Eppendorf) using 4uL of the synthesized cDNA and the iQ SYBR Green SuperMix (BioRad). The expression of genes of interest were quantified and normalized to house keeper genes. Data was normalized, quantified, and analyzed using Array Studio software (Omicsoft).

4.17 Luciferase Assay

A key aspect of this research was to focus on high throughput screening of small molecules that could direct differentiation of pluripotent cells to a cardiac fate. However, the previously described methods of high content screening and quantitative reverse-transcriptase

PCR were found to be too slow, detail oriented, and costly to achieve the desired results. Therefore, a luciferase assay was developed to serve as high throughput and relatively lower cost method of quantifying the cardiomyocyte gene, αMHC. hiPS were cultured following previous methods and 2-D differentiation was carried out on matrigel using, either BMP2 and AA or BMP4 and AA. After the mesoderm differentiation phase cells were plated into 96 well cell culture plates with white bottoms (Greiner) for luciferase assay. Wells were selected for either transduction or control. Transduction wells were transduced with a 30% volume to volume ratio of the lentivirus reporter in StemPro 34 media to a total volume of 100uL. After a 12 hour incubation at 37C with the lentivirus, the cells were washed three times with fresh StemPro 34 media. 100uL of fresh media was placed in each well of a 96 well plate. On day 7 of the differentiation experiment, inhibitors were added to experimental wells in a concentration of 1uM, 300nM, or 100nM. The inhibitors were diluted in StemPro 34 and a total concentration of 1% DMSO was present. For controls 1% DMSO in StemPro 34 was added as the vehicle. On day 9, the inhibitor application was removed and the cells were washed three times with StemPro34 media. hiPS were grown until day 14 with fresh StemPro 34 media being added every 24 hours. On day 28 cells were removed from the incubator and allowed to equilibrate to room temperature to ensure a uniform luciferase signal. Following the manufacturer protocol, a SteadyGlo solution (Promega) was made. 100uL of SteadyGlo luciferase detection buffer was added to each well of the 96 well plate on top the culture media. hiPS were incubated for 5 minutes before reading assay on a Viewlux (PerkinElmer) to detect luminescence. Luminescence data was exported by the Viewlux to a notepad file with raw counts per well. Data was quantified and analyzed for significance using GraphPad 5 (Prism).

CHAPTER 5: RESULTS

5.1 mES Embryoid Body Formation is Dependent on Cell Number

Embryoid body (EB) formation was used to induce differentiation of mES. This method has been demonstrated in the literature to induce the differentiation process of mES. When in suspension these cells will spontaneously form 3-D structures that resemble an embryo (Kurosawa,2007). In this current study, the hanging drop method was employed. This method employs supplying a specific number of mES in a 20uL drop and inverting the plate to hang cells in suspension (Diagram 1). It was found that cell density present in the hanging drop contributed to the cells' ability to form an embryo like structure. EBs that were plated with a higher cell density, 2,000 cells/EB, did not readily form one embryoid body but rather formed a mid-sized embryoid body with several smaller satellite EBs (shown in figure1). This is not a desired result for differentiation experiments because the satellite EBs have the potential to form independent signaling networks which have the potential to secrete signaling molecules that will bias the experiment that is not accounted for with one large EB. It was found that EBs seeded with 1,500 cells/ drop created one solid EB as demonstrated in figure2. Thus further experimentation was carried out with 1,500 cell EBs.

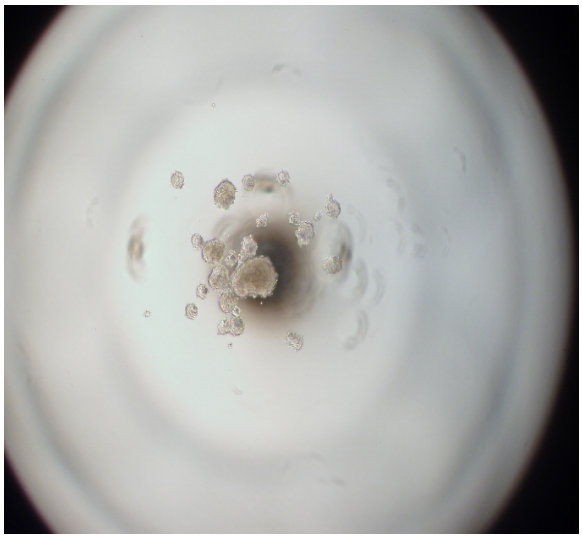


Figure 1. Embryoid Body formed with 2K Cells.

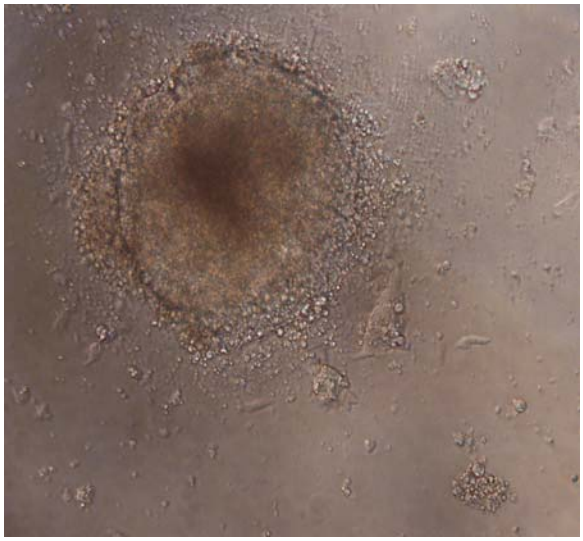


Figure 2. Embryoid Body formed with 1.5K Cells.

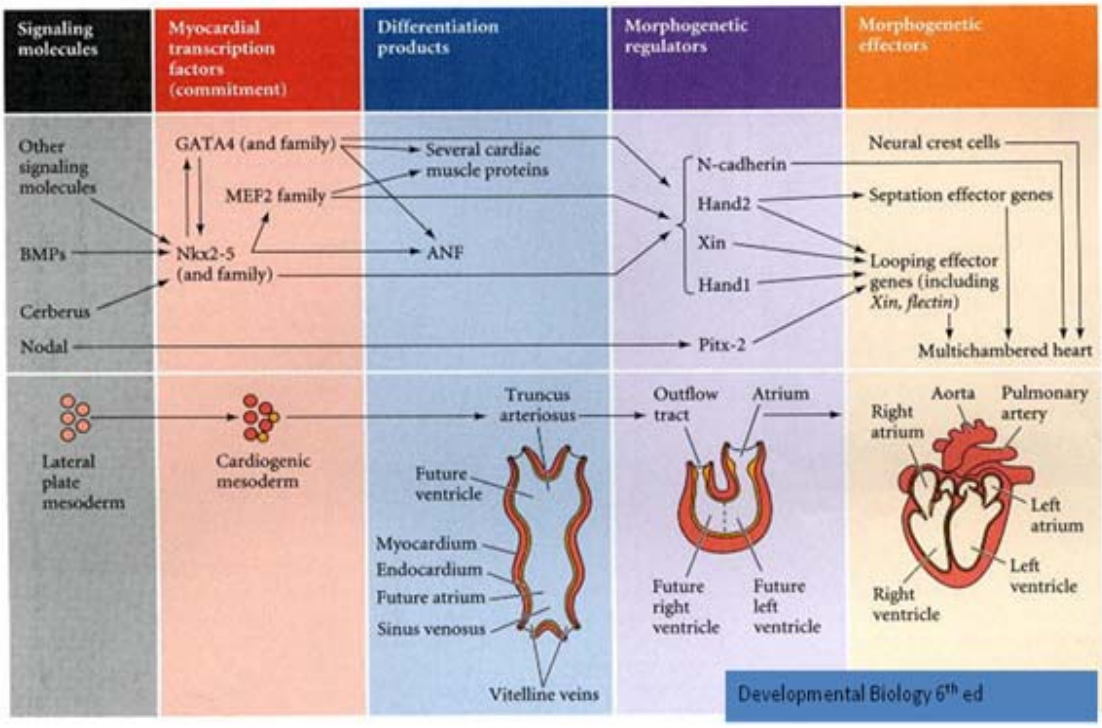


Figure ii. Signaling Pathways Involved in Cardiogensis.

5.2 mES Embryoid Body Formation Leads to the Formation of Cardiogenic Mesoderm

To follow a known marker of cardiac mesoderm, the MEF2C gene (Martin-Puig *et al*, 2008), the mES cell line obtained was stably transfected with a MEF2C reporter driving dsRed. As demonstrated in Figure ii, the MEF2 family of genes are activated early in cardiogenic mesoderm formation. The EBs and dissociated EBs were observed using the Perkin Elmer Operretta for dsRed activity. By day 5 of the differentiation experiments, MEF2C activation is observable in whole EBs however it is not clearly active in dissociated EBs that have been re-plated at different densities (observed in figures 3-5). The marker remains activated throughout early differentiation in whole EBs as seen in figure 6, whole EB seeded wells at day 10. Quanitifying the dsRED marker proved difficult using a high content imaging platform. These plateforms rely on a monlayer of cells and reporters with strong signals. However, the mES cell

culture did not remain in a monolayer throughout the course of the differentiation experiments and formed large 3-d cultures. The reporter also demonstrated a very weak signal. A MEF2C antibody and immunofluourescence chemistry was also employed but quantification remained intangible as seen in figure 7.

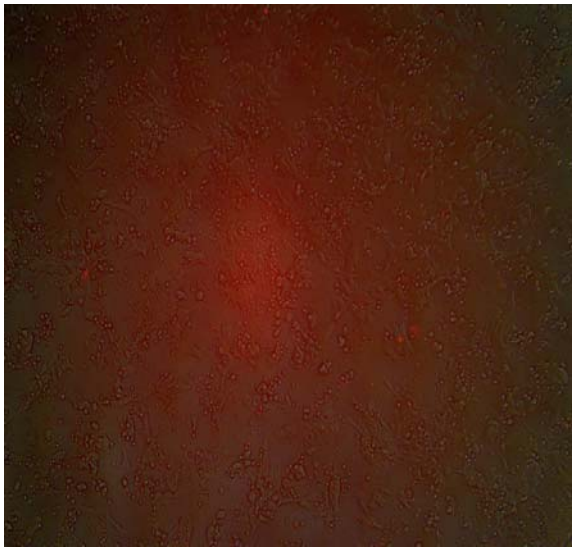


Figure 3. 40K mES cell per well Dissociated Embryoid Body.

Figure 4. 12K mES cell per well, Dissociated Embryoid Body.

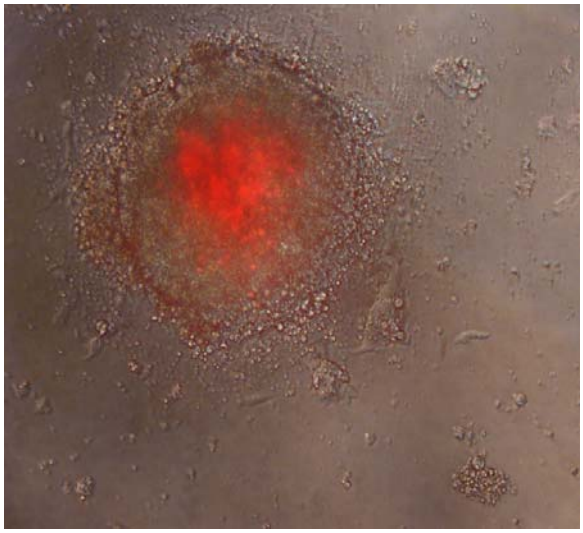


Figure 5. 1.5K mES cells, Embryoid Body Day 6 of Differentiation. MEF2C Reporter detectable.

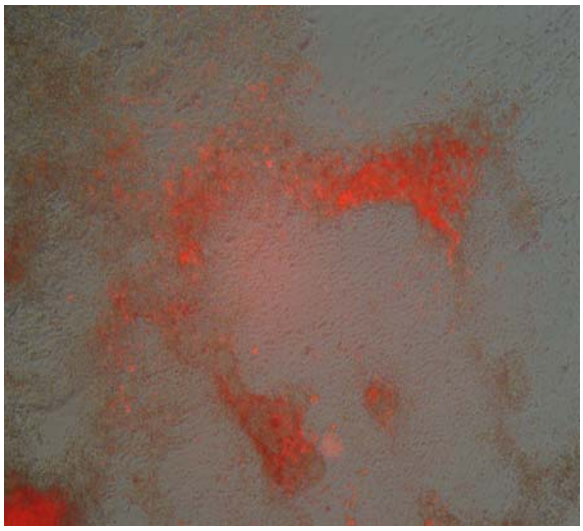


Figure 6. 1.5K mES cells, Embryoid Body Day 10 of Differentiation.

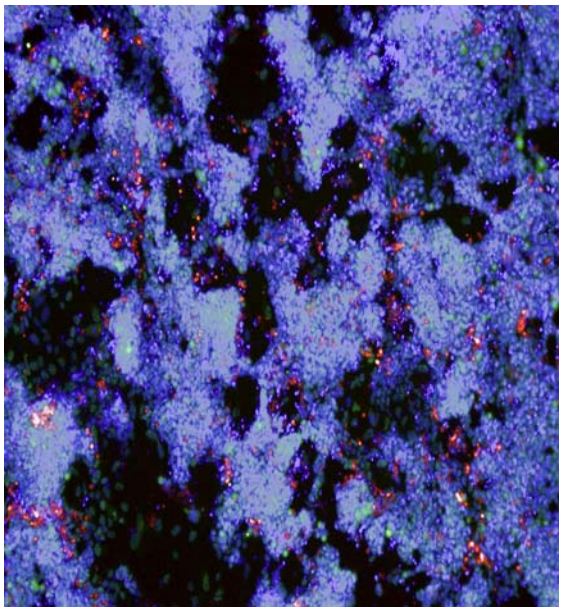


Figure 7 . Whole EB, Day 10. MEF2C reporter is detectable. Cells permeablized and stained with MEF2C antibody.

5.3 mES Embryoid Body Formation Leads to Spontaneous Cardiogenesis

15 days after EB formation from the hanging drop method, various beating areas were observed in the 96 well plates that contained whole EBs (See supplementary Video 1). However, colonies of EBs that were dissociated and re-plated at various densities, from 3K to 40K cells/well, did not demonstrate beating regions. qRT-PCR also demonstrated that 3 genes associated with cardiomyocytes were expressed in wells containing whole EBs as seen in figure 8. This suggests that EB formation from the hanging drop method does lead to cardiogenesis. Therefore, it was concluded that the hanging drop method is sufficient to induce differentiation of mES to mesoderm and cardiac tissue. As a consequence, the hanging drop method was employed for further differentiation experiments and compound screening.

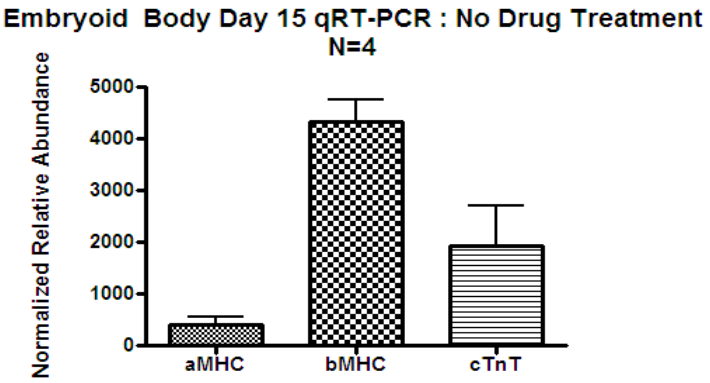


Figure 8. qRT-PCR results of whole mES Embryoid Body at day 15 of Differentiation.

5.4 ALK5 Inhibition Increases the yield of mES that Commit to a Cardiomyocyte Fate

It was hypothesized from the observations in a study published by Sridurongrit *et al.* that inhibiting ALK5 during development could increase the yield of mES that commit to cardiomyocytes. Sridurongrit *et al.* reported that ALK5's role in myocardium development is redundant however plays a critical role in epicardium and endocardium development. These

findings further suggested that knocking out ALK5 during development could lead to the activation of alternate signaling cascades that favor the development of cardiac muscle over vascular tissue and other other cell types associated with the heart (Sridurongrit *et al*, 2008). Thus, inhibiting ALK5 activity expression after mesoderm development was hypothesized to possibly increase cardiomyocyte yield from mES. Both dissociated EBs (3K, 10K, 20K cells/well) and whole EBs were tested against a vehicle control and various concentrations of a known specific ALK5 inhibitor. Experimental populations were dosed with the ALK5 inhibitor for 3 days between days 7 to 9 of development. Experimental design can be viewed in Design 1. qRT- PCR revealed that in whole plated EBs, ALK5 inhibition at a concentration of 1uM did significantly ($p > 0.05$ for all three genes) increase the relative abundance of three genes (cTnT, αMHC, βMHC) associated with cardiomyocytes when compared to vehicle control, as shown in figure 9a-c, significance demonstrated in Table 1. All data was normalized to GAPDH before analysis. ALK5 inhibition at 300nM and 3uM did not increase cardiogenesis when compared to vehicle. The application of the ALK5 inhibitor at 1uM significantly increased cardiogenesis, as shown in Table 1d., when compared to the other test concentrations of 300nM and 3uM. Thus, ALK5 inhibition's effect on cardiogenesis occurred in a dose dependent manner in these experiments. cTnT expression was also identified by IFC on the Perkin Elmer Operetta in whole EBs dosed with 1uM ALK5 inhibitor, figure 10. cTnT is seen in a long network of bundles typical to cardiomyocyte morphology. Further, visibly beating areas were also observed throughout the whole EBs treated with 1uM ALK5 inhibitor (see supplementary Video 2).

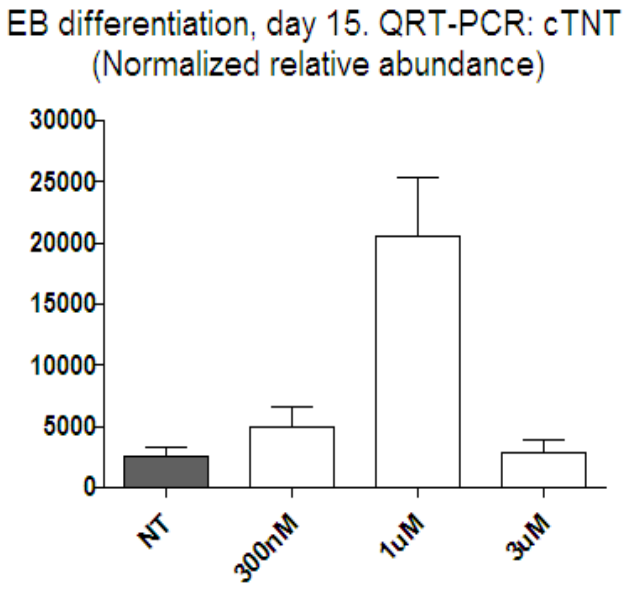


Figure 9a. cTnT. mES cells plated as whole EBs, day 15 of differentiation after ALK5i Treatment.

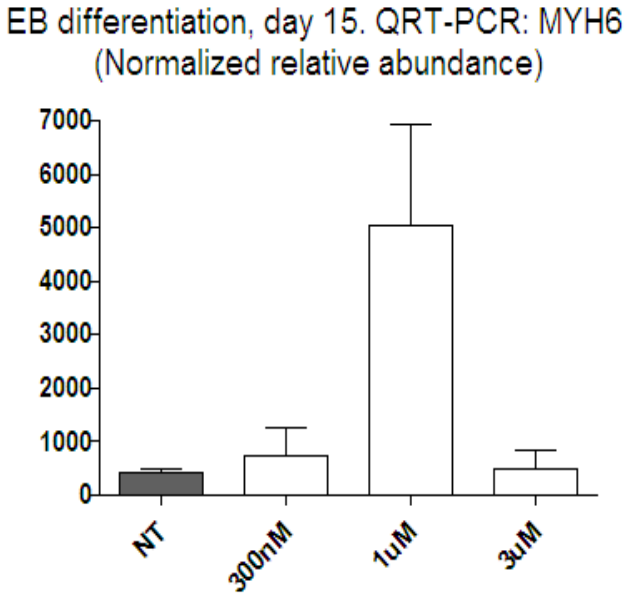


Figure 9b. . α-MHC. mES cells plated as whole EBs, day 15 of differentiation after ALK5i Treatment.

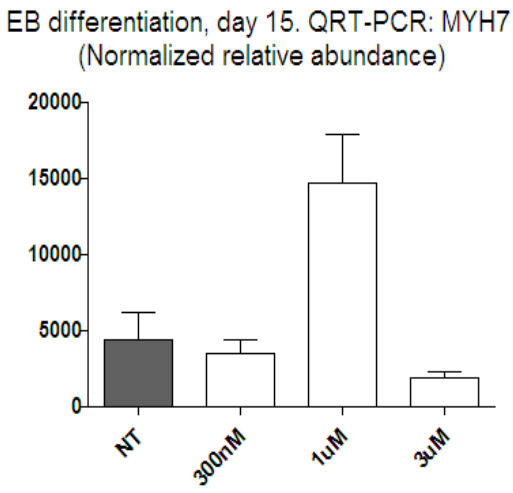


Figure 9c. βMHC. mES cells plated as whole EBs, day 15 of differentiation after ALK5i Treatment.

Table 1. Statistical Analysis of Cardiac Markers in mES and Dose Dependence.
1a. Statistical Analysis of Cardiac Troponin T expression in ALK5 inhibited mES.
1b. Statistical Analysis of alpha Myosin Heavy Chain expression in ALK5 inhibited mES. **1c.** Statistical Analysis of beta Myosin Heavy Chain expression in ALK5 inhibited mES.
1d. Statistical Analysis of Cardiac Troponin T expression in ALK5 inhibited mES and the effect of Dose Dependence.

Gene: cTnT

Parameter	300nM	1uM	3uM
Whole EB	EB	EB	EB
Control	NT	NT	NT
vs	vs	vs	vs
Experimental ALK5i Dose	300nM	1uM	3uM
Unpaired t test			
P value	0.2331	0.0103*	0.7823
Are means signif. different? (P < 0.05)	No	Yes	No
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed

Table 1a.

Gene: aMHC

Parameter	300nM	1uM	3uM
Whole EB	EB	EB	EB
Control	NT	NT	NT
vs	vs	vs	vs
Experimental ALK5i Dose	300nM	1uM	3uM
Unpaired t test			
P value	0.5748	0.0474*	0.7841
Are means signif. different? (P < 0.05)	No	Yes	No
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed

Table 1b.

Gene: bMHC

Parameter	300nM	1uM	3uM
Whole EB	EB	EB	EB
Control	NT	NT	NT
vs	vs	vs	vs
Experimental ALK5i Dose	300nM	1uM	3uM
Unpaired t test			
P value	0.6542	0.0283*	0.217
Are means signif. different? (P < 0.05)	No	Yes	No
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed

Table 1c.

Gene: cTnT		
Parameter	300nM vs 1uM	3uM vs 1uM
Whole EB	EB	EB
Experimental ALK5i Dose	300nM	1uM
vs	vs	vs
Experimental ALK5i Dose	1uM	3uM
Unpaired t test		
P value	0.022*	0.0116*
Are means signif. different? (P < 0.05)	Yes	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed

Table 1d.

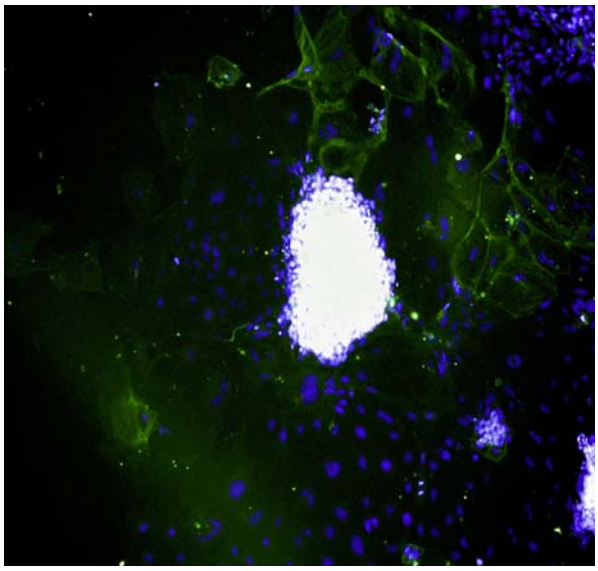


Figure 10. mES Day 11 of differentiation, 1uM ALK5i treatment. cTnT expression identified by antibody staining (Green). Nucleus Identified with Hoechst (Blue) .

It was also displayed that inhibition of ALK5 signaling in dissociated EBs did not lead to any significant rises in cardiogenesis when compared to their vehicle controls, as shown in figure 11.

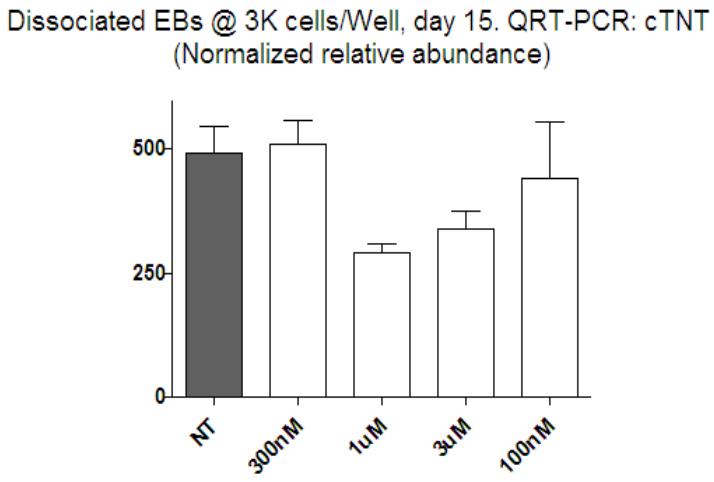


Figure 11. cTnT. mES cells plated as dissociated EB 3K, day 15 of differentiation after ALK5i Treatment.

5.5 2-D Culture is suitable for Human Induced Pluripotent Stem Cell (hiPS) Differentiation/ Activin A and BMP-2 Treatment produce Cardiac Tissue

It was discovered early in the work performed with hiPS that they do not readily form embryoid bodies like mES. Thus, another method of beginning differentiation needed to be devised. A 2-d culture system was developed using matrigel. Matrigel forms networks of proteins that the hiPS attach to and form colonies throughout. However unlike mES stem cells, these cells do not randomly differentiate readily. To overcome this shortcoming of hiPS, 6 protocols employing known signaling molecules associated with mesoderm formation were employed (Filipczyk *et al*, 2007). Combinations of Activin A, BMP-2, BMP-4, and WNT-3 were applied to the cultures for the first 5 days of the differentiation experiments. Details for 4 of the 6 protocols remain confidential due to intellectual property issues, thus the results are presented but the methods are removed. To compare the 6 protocols and their ability to produce cardiogenic mesoderm, cells were grown 10 days out after differentiation protocol, for a total of 15 days differentiation. Cells were lysed and qRT-PCR was employed to quantify 3 cardiac markers. All data was normalized to GAPDH, a house keeper gene, before analysis. Protocol 1 was found to

produce the largest quantity of Cardiac markers consistently. This protocol employed BMP-2 and Activin A in equal amount for 5 days of treatment. Protocol 4 was also found to produce large quantities of cardiac markers. This protocol lead to a large production of cTnT, a canonical marker of maturing/mature cardiomyocytes (Martin-Puig *et al*, 2008). This protocol engaged BMP-4 and Activin A in equal amounts for 5 days of treatment. Results are shown in figure 12. Thus, these two mesoderm differentiation protocols were used for further differentiation experiments.

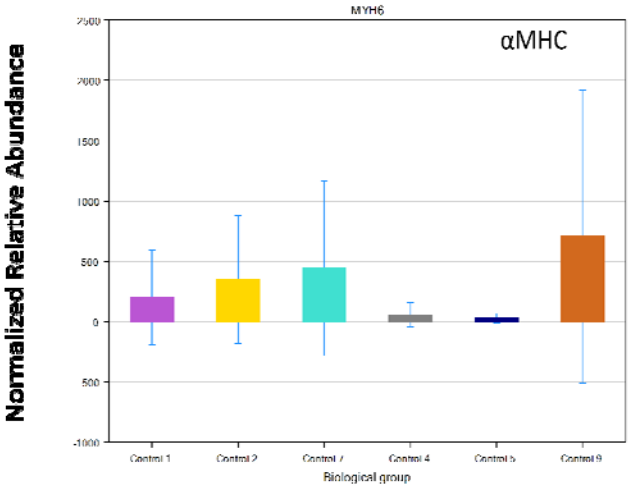


Figure 12a. Quantification of α-MHC in hiPS for the 6 mesoderm protocols at day 15.

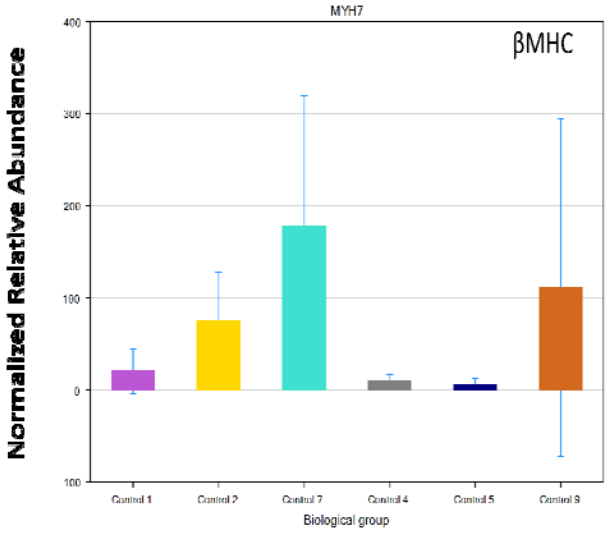


Figure 12b. Quantification of β-MHC in hiPS for the 6 mesoderm protocols at day 15.

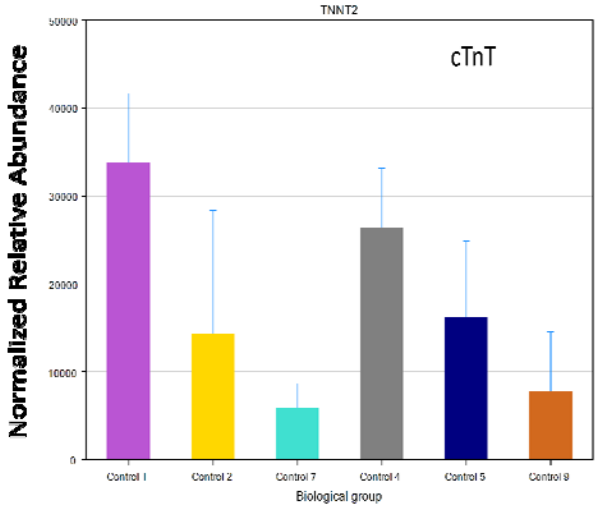


Figure 12c. Quantification of cTnT in hiPS for the 6 mesoderm protocols at day 15.

5.6 ALK5 Inhibitor identified in mES model to Increase Cardiogensis did not repeat in hiPS Model

hiPS were grown on matrigel following protocols listed in the methods section. hiPS were then differentiated on matrigel using the 2-d culture and signaling factors discovered in the previous experiment. On day 7 of the differentiation experiment, 2 days post mesoderm

induction, hiPS were treated with the ALK5 inhibitor identified in the mouse stem cell screen. hiPS were treated with 1uM, 300nM, 100nM, or vehicle control of 1%DMSO in fresh growth media. This treatment was for 72 hours. After 72 hours, the wells were washed and fresh media was added daily until day 28 when cells were lysed for qRT-PCR. The results obtained from qRT-PCR demonstrated that the increase of cardiogenesis observed in mES did not replicate in the hiPS model. Figure 13 demonstrates these results, all experiments were performed for an N = 8.

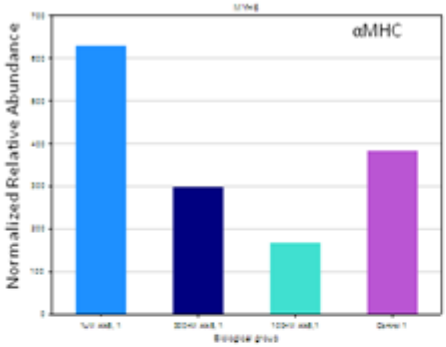


Figure 13 a.

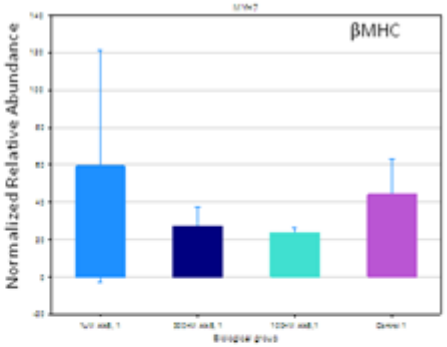
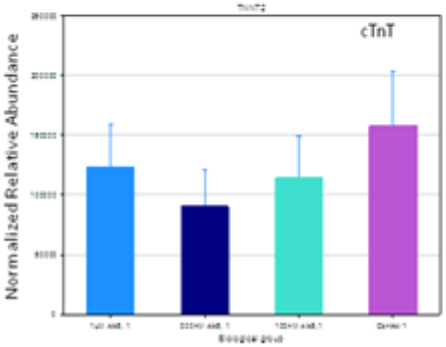


Figure 13 b.



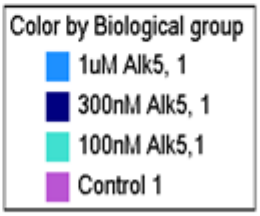


Figure 13 c.

Figures 13a-c. Differentiation experiment with ALK5i in hiPS. a) Quantification of α-MHC at day 28. b) Quantification of β-MHC at day 28. c) Quantification of cTnT at day 28.

5.7 ALK5, p38, or JAK/STAT Inhibition did not increase Cardiogenesis

Given that the ALK5i identified from the mES screen did not increase cardiogenesis, this experiment was repeated using an ALK5 inhibitor from a different chemical series. Also, following work performed by Graichen *et al*., a p38 MAPK specific inhibitor was chosen from the compound collection. Graichen *et al.* demonstrated in human embryonic stem cells that inhibiting this pathway during early differentiation lead to significant increases in α-MHC production when compared to controls (Graichen *et al*, 2008) (see figure 14). Further the role of the JAK/STAT pathway was also explored. For this round of experiments, hiPS were grown following previously mentioned protocols and mesoderm was induced using Activin A and BMP2. On day 7 of the differentiation experiments media was supplemented with either 1uM ALK5, p38, or JAK/STAT inhibitor, or 1%DMSO as the vehicle control. This treatment was for 72 hours. After 72 hours, the wells were washed and fresh media was added daily until day 28 when cells were lysed for qRT-PCR. The results do not demonstrate any significant rise in cardiomyocyte markers when compared to vehicle controls as shown in figure 15. All experiments were performed for an N = 8.

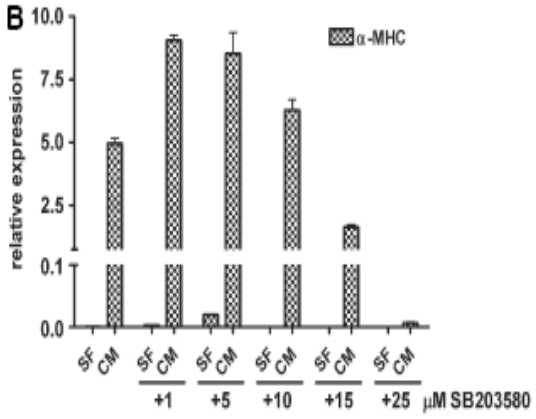
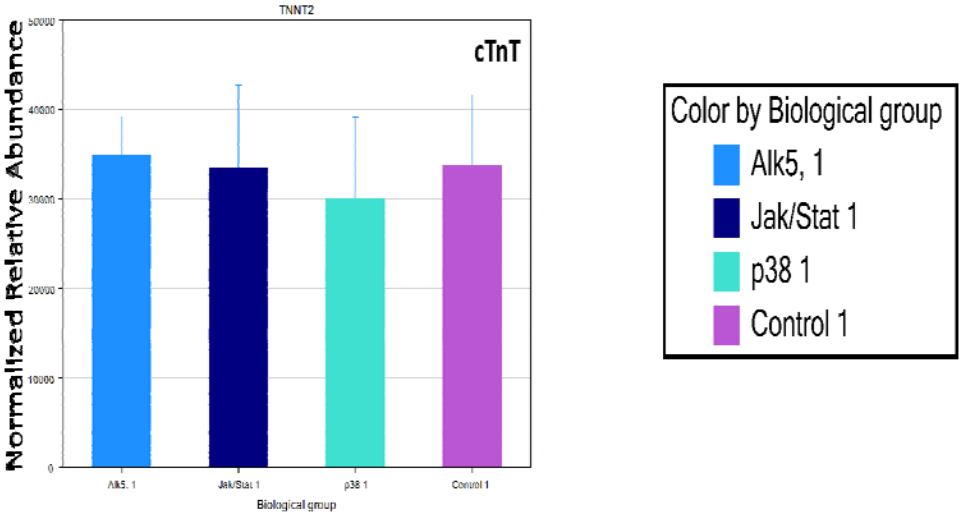


Figure 14. Expression of α-MHC from a dose response of a p38 inhbitor vs vehicle. In serum free media (SF) and ENDO-CM media.



Figures 15. Differentiation experiment with various pathway inhibitors in hiPS. Quantification of cTnT for at day 28.

5. 8 Luciferase assay and Lentiviral reporter can detect Cardiac Troponin T (cTnT) in Appreciable Amounts

To ensure that the lentivirus reporter for cTnT driving luciferase was functioning, mature cardiomyocytes obtain from Cellular Dynamic International (CDI) were transduced with the reporter in a titer. iCell cardiomyocytes were obtained at day 30 from CDI and grown in culture until day 60, the time point cells reach full maturity according to manufacturer protocol. At day 60, the cardiomyocytes were transduced with 100%, 33% or 3% lenti (volume lenti relative to total volume media) or left uninfected (each condition in triplicate). It was found that ~30% volume to volume transduction produced the highest luminescent signal from cTnT production, figure 16. Thus, the luciferase assay in this report is run at 30% lentivirus for a 12 hour transduction.

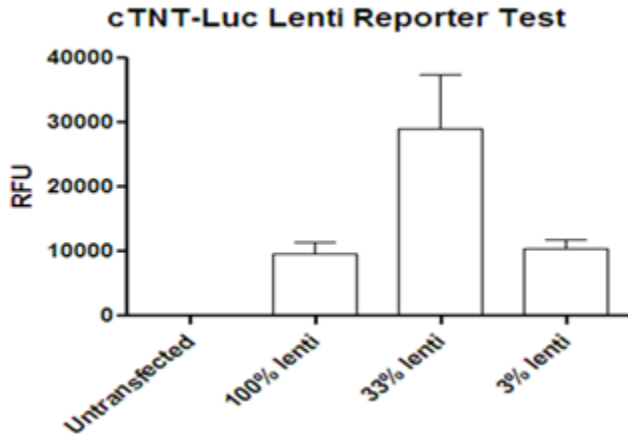


Figure 16. cTnT production, quatinfied by luciferase Reporter in iCell Cardiomyoctes from CDI.

5.9 Luciferase assay and Lentiviral reporter screening did not reveal Small Molecules that Positively directed Differentiation to a Cardiomyocyte Fate

Given that the qRT-PCR assay did not reveal any successful molecules, the compounds were repeated and expanded in the luciferase assay. hiPS differentiation began with Activin A and BMP2 or BMP4 following previously listed protocols. Both mesoderm protocols were compared in the assay to determine their effect on cardiogenesis with small molecule screening. The first round of differentiation experiments explored the application of the ALK5 inhibitor identified in the mES screen in a dose curve of 100nM, 300nM, 1uM. This protocol was repeated using two other non specific ALK4/5 inhibitors from different chemical series. Cells were allowed to mature to day 28. This experiment did not yield any positive outcome on cardiogenesis when compared to spontaneous differentiation in the hiPS cell line, as shown in figure 17.

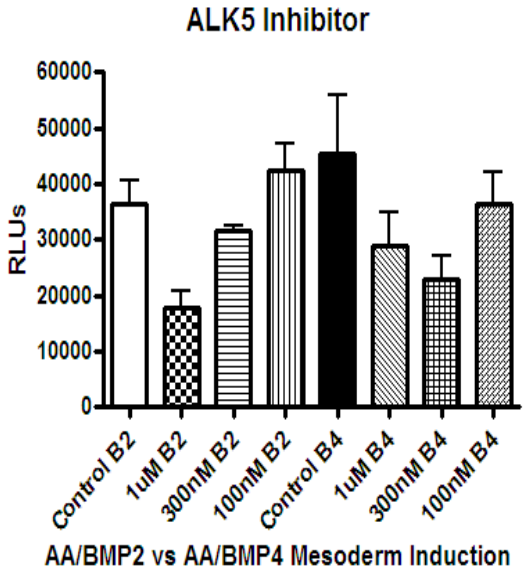


Figure 17a. Quantification of cTnT in hiPS after the application of the ALK5 specific inhibitor identified in the mES screen.

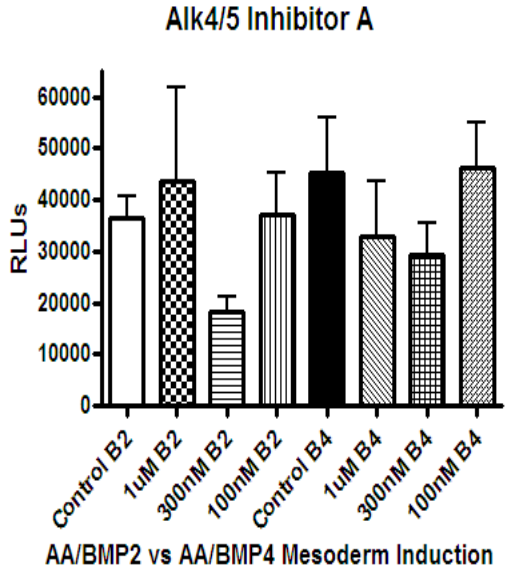


Figure 17b. Quantification of cTnT in hiPS after the application of a ALK4/5 specific inhibitor.

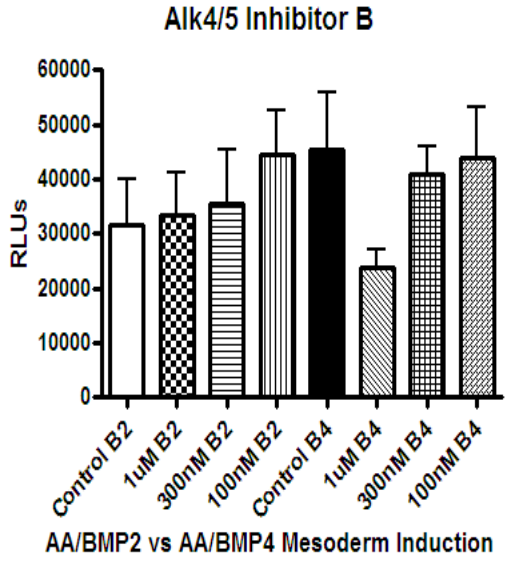
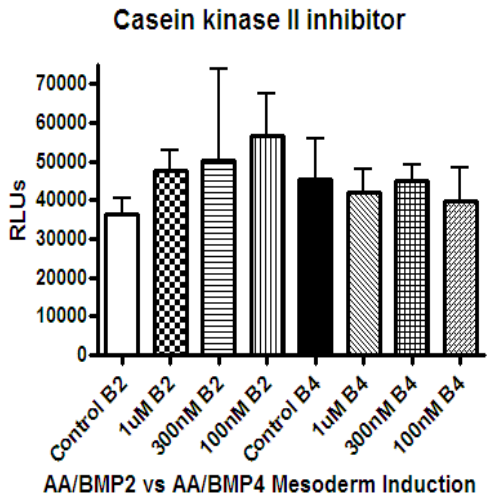


Figure 17c. Quantification of cTnT in hiPS after the application of a ALK4/5 specific inhibitor.

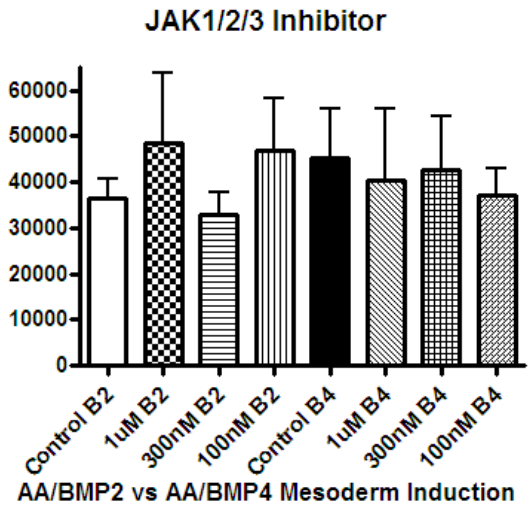
The luciferase assay was also performed to study the effect of casein kinase 2 inhibition. This kinase has been shown to be a negative regulator of BMP2 (Bragdon *et al*, 2010). Inhibition of this enzyme could potentially up-regulate BMP signaling and increase cardiogenesis. The dose curve in both mesoderm protocols did not demonstrate any significant increases in cTnT when compared to controls, shown in figure 18. The JAK/STAT pathway was also explored using a broad acting inhibitor of JAK1/2/3. This experiment did not yield any significant increase in cTnT expression at 28 of the differentiation experiment, shown in figure 19.

Inhibition of the ROCK kinase was also explored for effect on cardiomyocte levels in differentiation experiments. This experiment did not yield any significant increase in cTnT expression at 28 of the differentiation experiment, shown in figure 21.

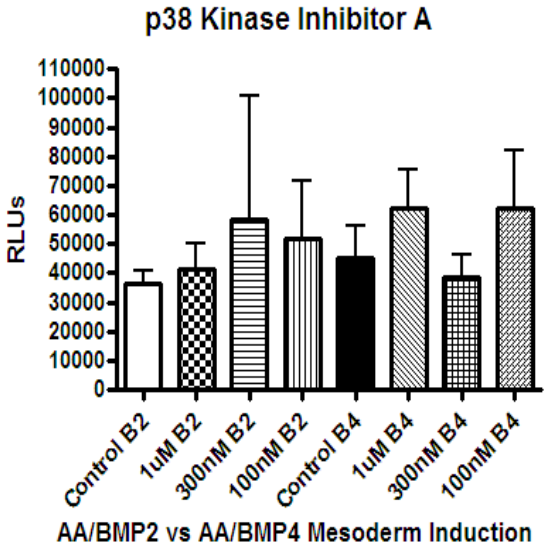


Figures 18. Luciferase assay with hiPS comparing Mesoderm induction protocols and Casein Kinase II inhibition, Day 28.

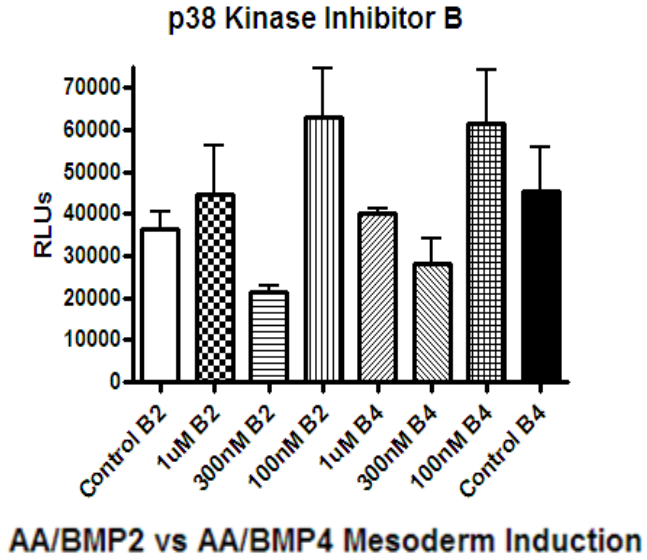
The final luciferase assay performed focused on three specific p38 kinase inhibitors.This experiment was to test the data presented by Graichen *et al*, in which p38 inhibition lead to significant increase of α-MHC expression when compared to spontaneously differentiated control embryonic stem cells. It was shown that p38 kinase inhibitor B did lead to significant increase in cardiogenesis at 100nM application in the Activin A and BMP2 induction protocol (p=0.038). This significance was not replicated in the Activin A and BMP4 induction protocol (p=0.39). Neither the p38 inhibitor A or C produced significant increases of cTnT, data shown in figure 20.



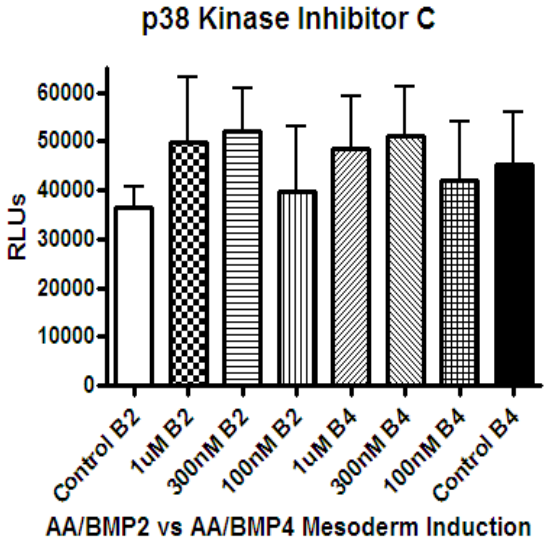
Figures 19. Luciferase assay with hiPS comparing Mesoderm induction protocols and JAK/STAT inhibition, Day 28.



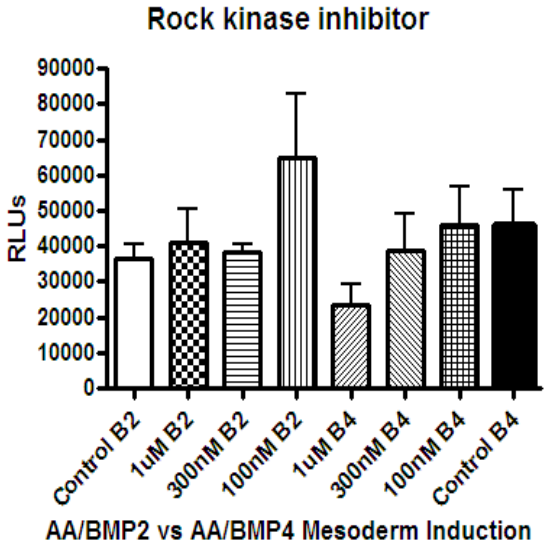
Figures 20a. Luciferase assay with hiPS comparing Mesoderm induction protocols and p38 inhibition A, Day 28.



Figures 20b. Luciferase assay with hiPS comparing Mesoderm induction protocols and p38 B inhibition, Day 28.



Figures 20c. Luciferase assay with hiPS comparing Mesoderm induction protocols and p38 C inhibition, Day 28.



Figures 21. Luciferase assay with hiPS comparing Mesoderm induction protocols and ROCK inhibition, Day 28.

CHAPTER 6: DISCUSSION

The current study confirms previous work that small molecules can be used to direct differentiation of pluripotent stem cells. The mES model demonstrated significant increases in cardiogenesis through the inhibition of ALK5. Further, this successful experiment was not able to replicate in the currently studied hiPS cell line. This study also established three assays for studying the effect of small molecules on the differentiation process of cardiogenesis. qRT-PCR proved to a valuable tool in establishing initial cardiomyocyte levels in early assay development. High content screening proved to be unreliable to quantify cardiogenesis from antibody staining or reporter systems. The luciferase reporter system proved to be the most efficient as far as time and capital are concerned. This assay was used to screen a small population of small molecules with specific targets to determine their effect on cardiogenesis in hiPS. This assay can be readily duplicated to provide a high throughput format to screen unbiased small molecule compound screens have the potential to affect cardiogenesis either positively or negatively. This has the potential to reveal novel signaling pathways that have not been previously implicated in cardiogenesis.

The mES cells were readily able to form embryoid bodies of 1500 cells/EB; this demonstrated to be crucial in forming beating cardiomyocytes. Embryoid body formation proved to be a reliable protocol to begin differentiation of mES and induce random cardiogenesis. EB formation lead to the expression of two genes specifically associated with cardiomyocytes, cTnT and αMHC (Martin-Puig *et al*, 2008). Experiments involving dissociated EBs did not produce a comparable baseline of cTnT when compared to whole embryoid bodies. 20K cells/ well of dissociated EBs provided the highest level of cTnT, however whole EB wells with far fewer cells (1500 to 3000) provided a baseline of normalized cTnT mRNA expression of more than double this level. Therefore this data suggests that signaling pathways established during early and late stage embryoid body formation and differentiation remain crucial to successful cardiogenesis and

cardiomyocyte formation. However, EB formation only leads to small detectable beating regions of the tissue culture wells. This observation justifies the need for more robust protocols to increase cardiogenesis from pluripotent cells. Future experimentation and microarray analysis of these whole EBs could reveal these critical signaling pathways and provide researchers with future targets for chemical modulation to increase cardiogenesis in large dissociated populations of EBs from pluripotent cells.

The mES cell model also proved to be successful in the first compound differentiation experiment, adding evidence to the hypothesis of ALK5 signaling in cardiomyocyte differentiation to be redundant. Though it has been established that the TGF-B super family plays a critical role in cardiac development (Sanford *et al*¸ 1997), Sridurongrit *et al* have suggested the role of Type I receptors, such as ALK5, did not play a role in cardiac muscle development (Sridurongrit *et al*, 2008). This research adds further evidence to support this hypothesis. The application of an ALK5 specific inhibitor did not decrease cardiogenesis from baseline induction via the EB formation route. In fact as suggested by the authors, inhibiting ALK5 lead to a significant increase of cardiogenesis. Providing positive data to their proposed hypothesis that knocking out ALK5 changed the signaling pattern normally observed during cardiac development towards a cardiomyocyte fate. Further experiments are needed to reveal if this increase of cardiomyocytes occurred at the expense of other cardiac tissue as suggested by the authors. To date to this author's knowledge, no data on ALK5 inhibition has been published to confirm or disprove the outcome of ALK5 inhibition positively affecting cardiogenesis from pluripotent cells. Further the inhibition of ALK5 leading to increases of cardiomyocytes was found to be dose specific. The inhibitor only produced a significant rise in cardiomyocyte markers when applied at 1uM. This concentration proved to be significant over not only the vehicle control but the other experimental concentrations as well. This could be the result of the lower concentration, 300nM, not inhibiting enough of the kinase, ALK5, to down regulate its normal function or the higher

concentration of 3uM could prove cytotoxic. This suggests that future differentiation experiments should run full dose response curves of compounds to determine how effective a small molecule will be in directing differentiation to a desired somatic fate.

This research found the employed hiPS cell line did not readily form embryoid bodies comparable to the mES cells. Therefore, another culture model was needed to induce differentiation. A simple literature search demonstrated that 2-d culture using matrigel is a suitable alternative for hiPS culture and differentiation. In the matrigel culture system, hiPS will readily differentiate but this was prevented through high concentrations of basic fibroblast growth factor (bFGF) being supplemented in the hiPS maintenance media (Ulloa-Montoya *et al*, 2005). Differentiation of a pluripotent cell occurs in two major stages: pluripotent (ie stem cell) to multipotent (ie cardiac mesoderm) to a terminally differentiate somatic cell (ie cardiomyocyte) (Hochedlinger *et al*, 2009). Therefore, protocols for each stage of differentiation were developed. To induce mesoderm, six different protocols that used combinations of Activin A, BMP2, BMP4 or WNT-3 were employed. These experiments demonstrated that Activin A in combination with either BMP2 or BMP4 produced the highest quantity of cardiac tissue by qRT-PCR analysis. This follows previous research which revealed BMP2 and BMP4 are co-expressed in similar temporal locations as GATA4 and Nkx2.5 in cardiac mesoderm (Schultheiss *et al*, 1997). The results of this study again imply these factors in combination with GATA4 and Nkx2.5 control the expression of cardiac transcription factors.

To study the second major stage of differentiation, mesoderm to cardiomyocyte, small molecule inhibitors of signaling pathways were tested. The ALK5 inhibitor that was identified to positively affect cardiogenesis in mES did not replicate in the hiPS cell line. Further exploration of non specific ALK5 inhibitors also produced no substantial increase in cardiomyocyte production over baseline in this research. Inhibition of the p38 kinase did not produce any appreciable effect on cardiogenesis over baseline in the hiPS differentiation experiments except in

one instance which needs confirmation. This is in contrast to the research performed by Graichen *et al,* in which p38 inhibition in human embryonic stem cell differentiation experiments produced significant amounts of α-MHC mRNA when compared to baseline spontaneous differentiation controls. Alternative p38 inhibitors were also tested with negative results. A casein kinase 2 inhibitor was also tested. This kinase has been identified as a negative regulator of BMP signaling (Bragdon *et al,* 2010). Given that BMP signaling is closely linked to cardiac development (Filipczyk *et al*, 2007), it was hypothesized inhibiting this negative regulator could have a positive outcome on cardiogenesis. However, this study once again did not produce any positive correlation between casein kinase 2 inhibition and cardiogenesis from hiPS. Further a Rho-associated kinase (ROCK) inhibitor was also tested. Recent research has demonstrated that ROCK activity plays a role in heart disease and inhibition of this kinase has also been identified in the survival of cardiomyocytes derived from human embryonic stem cells (Braam *et al*, 2010). Therefore, it was explored if inhibiting ROCK activity could increase the overall cardiogenesis of hiPS, this study did not reveal any positive correlation between ROCK inhibition and cardiomyocyte levels in this hiPS cell line. Finally a JAK/STAT inhibitor was tested, given this pathway is highly active in embryonic stem cells and cancer cells (Dreesen *et al*, 2007), it was thought inhibition could lead to more terminal differentiation and positively affect mesodermal precursors to differentiate into mature cardiomyocytes. However, none of the hiPS differentiation protocols employing small molecules demonstrated any significant increase in cardiogenesis.

Since ALK5 inhibition lead to significant increases in cardiogenesis in mouse embryonic stem cells and not human induced pluripotent stem cells (hiPS) there are several areas that need to be explored in the future. Firstly, the role of ALK5 signaling has not been identified in human cardiogenesis. To date only experiments with murine models have characterized ALK5 signaling in cardiac development. These studies have demonstrated that ALK5 signaling is critical to epicardium and endocardium development but not myocardium (muscle) development

(Sridurongrit *et al*, 2007). It is possible that myocardial development in humans requires ALK5 signaling, this should be explored in future work. Secondly, the temporal expression of this signaling pathway in human cardiogenesis is inadequately understood. It is possible that the timelines for drug application performed in mES do not accurately represent hiPS differentiation. Therefore, this research advocates the need for not only full dose testing of compounds for their impact on differentiation, but temporal studies of compound application will be required as well. Lastly, there are possibly genetic differences between each cell line that have not been explored in this research. Genotyping of every donor for differentiation experiments should be performed to reveal functional signaling pathways before small molecule testing. Thus, it is possible that each of these cells lines used possessed varying signaling capacity of the ALK5 pathway which skewed the data either positively or negatively.

There are also several complications when working (hiPS) that were not explored in this research. Firstly, recent work has suggested that the karyotypes of some induced pluripotent cell lines may demonstrate aneuploidy throughout passaging and other genomic errors that were not previously thought to exist (Mayshar *et al*, 2010). If long term expansion is associated with genomic instability in these cells, future work will have to monitor this throughout the differentiation experimentation to ensure reproducible results. Secondly, hiPS are reprogrammed through ectopic expression of oncogenes. Research has shown this oncogene expression can lead to DNA replication stress that ultimately creates genomic amplification and deletions (Pasi *et al*, 2011). If hiPS are to be successfully employed in drug screening or regenerative medicine, research will have to focus on developing reprogramming protocols that either mitigate these effects or use small molecules to avoid the over expression of oncogenes. Therefore it may be of priority of this new field of induced stem cells to focus on reprogramming before directing differentiation to ensure proper genomic stability of the cell lines. Lastly, research groups have begun to explore “epigenetic memories” retained during the reprogramming process of a somatic

cell to induced pluripotency. This research suggests that hiPS and other induced cell lines are skewed to return to the somatic fate from which they were derived. Research has begun to show that this epigenetic memory may be overcame through the application of small molecule inhibitors of the epigenetic machinery (Sullivan *et al*, 2010). Therefore it is possible the research in this paper would need to explore the epigenome of the hiPS cell line to ensure a truly "blank slate" before repeating differentiation experiments.

In conclusion, the future looks bright for pluripotent stem cell application in the fields of drug discovery and regenerative medicine given the recent breakthroughs. This research clearly demonstrated that embryonic stem cells can be successfully differentiated selectively but given their ethical implications the future will and should focus on induced pluripotent cell lines. However, the use of hiPS will require a much more extensive study and understanding of the reprogramming phase before directed differentiation will be successful.

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